A METHOD FOR GENERATING GENETICALLY ALTERED ANTIGENS

TECHNICAL FIELD OF THE INVENTION

The invention is related to the area of genetic alterations of antigens as potent

5   vaccines. In particular it is related to the field of mutagenesis.

BACKGROUND OF THE INVENTION

The use of vaccines to build immunity against foreign and/or endogenous polypeptides provides an effective and selective strategy for treating the underlying cause

10   of disease. In particular is the use of killed viruses such as the polio mellitus and the Hepatitis B virus (John T.J. (2000) *New Engl. J. Med.* 14:806-807). Standard methods for generating vaccines against candidate pathogenic organisms or molecules are known by those skilled in the art. Vaccines for human use are developed in animal models to survey for the ability of killed or defective whole agents such as parasites, viruses or recombinant

15   polypeptides to cause immunity against infection of the pathogenic agent (Boyce T.G. *et al.* (2000) *Vaccine* 19:217-226). Briefly, rodents such as mice or rats are injected with a purified antigen in the presence of adjuvant to generate an immune response (Boyce T.G. *et al.* (2000) *Vaccine* 19:217-226). Unfortunately, not all antigens are capable of eliciting a strong immune response when injected into a host organism (Hoshino Y. and A.Z.

20   Kapikian (2000) *J. Health Popul. Nutr.*18:5-14; Orenstein W.A. *et al.* (2000) *Am. J. Public Health* 90:1521-525; Lechmann M. and T.J. Liang (2000) *Semin. Liver Dis.* 20:211-226). While the reasons for the lack of immune response are not clear, some factors, such as the lack of T-cell epitopes which are important for stimulating cellular-mediated immune responses, may be absent within a given antigen (Ausiello C.M. *et al.*

25   (1999) *Infect. Immun.*67:4064-4071; Brosstoff S. (1995) *Adv. Exp. Med. Biol.* 383:249-254). In the case of parasitic infections, the development of effective vaccines has been hampered by the presence of many different developmental stages that occur within an infected host and that a diverse array of allelic forms occurs within genes encoding for prominent surface antigens (MALARIA OBSTACLES AND OPPORTUNITIES, Oaks, S.C. *et al.*,

Eds., National Academy Press, p 1, 1991; Anders, R.F. "Vaccines Against Asexual Blood Stages of *Plasmodium falciparum*" NEW GENERATION VACCINES, 2ⁿᵈ Ed., Anders, R.F., pp. 1035-1055, 1997). It is believed by many skilled in the art that the generation of highly antigenic polypeptides may overcome these limitations and produce a protective immune

5   response to pathogens (McLeod R. *et al.* (1995) *Curr. Opin. Immunol.*7:539-552).

A method for generating diverse sequences within a polypeptide would be useful for the creation of more potent therapeutic agents. Moreover, the generation of randomly altered nucleotides and encoded polypeptide residues throughout an entire antigen molecule may result in new reagents that are: 1) more antigenic; 2) more immunogenic; and 3) have

10   beneficial pharmacokinetic properties.

SUMMARY OF THE INVENTION

The invention described herein is directed to the use of random genetic mutation of a polypeptide *in vivo* by blocking the endogenous mismatch repair (MMR) activity of a host

15   cell yielding structurally altered antigens that can be screened for antigenicity and immunogenicity in comparison to the wild type molecule. The use of mammalian cell-based high throughput screens as taught by this application will facilitate identification of randomly altered antigens that may serve as effective vaccines. Moreover, the invention describes methods for repeated *in vivo* genetic alterations and selection for antigens with enhanced

20   immunogenicity and pharmacokinetic profiles.

The ability to develop and screen genetically altered mammalian cells that secrete structurally altered polypeptides in a high throughput manner provides a valuable method for creating vaccines for therapeutic development. A potential problem in generating potent vaccine antigens against endogenous to the mammalian host is the source of antigen

25   production. In many instances recombinant polypeptides that are naturally produced by mammalian cells are generated recombinantly using insect, yeast or bacterial expression systems. These sources typically produce large amounts of proteins that are distinct from the mammalian-produced polypeptides, and may differ from the natural protein due to altered folding or altered post-translational modifications such as hyperglycosylation. The invention

described herein is directed to the creation of genetically altered mammalian cell hosts that produce structurally altered polypeptides as vaccine agents via the blockade of MMR.

The present invention facilitates the generation of highly antigenic polypeptides as vaccines. The advantages of the present invention are further described in the examples and

5   figures described herein.

The present invention provides methods for generating genetically altered antigens *in vivo*, whereby the antigen possesses desired biochemical property(s), such as, but not limited to, increased antigenicity and immunogenicity. One method for identifying antigens with increased antigenicity is through the screening of mismatch repair ("MMR") defective cell

10   clones that produce desired antigens.

The invention also provides methods for rendering cells expressing a target antigen hypermutable. The cells include, but are not limited to rodent, primate, human, plant, yeast or bacterial cells. The antigens can be generated from endogenous genes or from introduced transgenes.

15   The invention also provides methods for generating genetically altered cell lines that express antigenic polypeptides.

In some embodiments, the invention provides methods for generating genetically altered cell lines that produce immunogenic polypeptides.

In other embodiments, the invention provides methods for producing an antigen

20   expression cassette for high throughput screening of altered polypeptides *in vivo*.

In other embodiments, the invention provides methods of mutating a gene of interest in a mismatch repair defective cell.

In some embodiments, the invention provides methods of creating genetically altered antigens *in vivo* by blocking the MMR activity of the cell host.

25   Still other embodiments of the invention provide methods of creating genetically altered polypeptides *in vivo* by transfecting genes encoding for an antigen in a MMR defective cell host.

The invention also embraces methods of creating antigens with increased immunogencity due to genetic alterations within the antigen-encoding gene by blocking endogenous MMR of the cell host.

In some embodiments, the invention provides methods of creating a library of randomly altered antigens from mammalian cells by blockade of MMR of the cell host.

In other embodiments, the invention provides methods of creating antigens with enhanced pharmacokinetic profiles due to genetic changes within the encoding gene by blocking endogenous MMR of the cell host.

The invention also provides methods of creating genetically altered antigens in MMR defective cells as vaccine agents.

In some embodiments, the invention provides methods for high throughput screening of antigens produced by MMR defective cells.

These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention, a method for making MMR defective cell lines expressing a target antigen will be provided. A polynucleotide encoding a dominant negative allele of an MMR gene is introduced into a target antigen-producing cell. The cell becomes hypermutable as a result of the introduction of the gene.

In another embodiment of the invention, an isolated hypermutable cell producing antigenic peptides is provided. The cell is defective for mismatch repair and exhibits an enhanced rate of hypermutation. The cell produces a polypeptide from a mutated gene encoding for the polypeptide.

In another embodiment of the invention, a method is provided for introducing a mutation into an endogenous gene encoding for a target polypeptide. A polynucleotide encoding a dominant negative allele of a MMR gene is introduced into a cell. The cell becomes hypermutable as a result of the introduction and expression of the MMR gene allele. The cell further comprises a gene of interest. The cell is grown and tested to determine whether the gene encoding for a polypeptide of interest harbors a mutation.

In another embodiment of the invention, a method is provided for producing a cell-based screening assay to identify antigenic proteins as vaccines. A polynucleotide encoding a

dominant negative allele of a MMR gene is introduced into a cell expressing a secreted antigen. The cell becomes hypermutable as a result of the introduction of the gene. The cell is grown and conditioned medium from the cell is tested for the expression of antigenic polypeptides.

5   In another embodiment of the invention, a gene, or set of genes encoding for polypeptides or a combination therein, are introduced into a mammalian cell host that is defective in MMR. The cell is grown and clones are analyzed for antigens with enhanced antigenicity.

In another embodiment of the invention, a method is provided for producing a cell-

10   based screening assay to identify antigenic proteins as vaccines. A polynucleotide encoding a secreted antigen is introduced into a naturally MMR defective cell. The gene is hypermutable as a result of the introduction of MMR deficiency. The cell is grown and conditioned medium from the cell is tested for the expression of antigenic polypeptides.

In another embodiment of the invention, a method will be provided for restoring

15   genetic stability in a cell containing a polynucleotide encoding for a dominant negative allele of a MMR gene. The expression of the dominant negative MMR gene is suppressed and the cell restores its genetic stability including but not limited to genetic stability within the antigen-encoding genes.

In another embodiment of the invention, a method will be provided for restoring

20   genetic stability in a cell containing a polynucleotide encoding a dominant negative allele of an MMR gene and a newly selected phenotype. The expression of the dominant negative mismatch repair gene is suppressed and the cell restores its genetic stability and the new phenotype is stable.

These and other embodiments of the invention provide the art with methods that can

25   generate enhanced mutability in cells and animals as well as providing cells and animals harboring potentially useful mutations for the large-scale production of highly antigenic polypeptides as potent vaccines.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: *In situ* β-galactosidase staining of TKPMS134/pCAR-OF or TKvect/pCAR-OF cells to assay for MMR defective cells containing genetically altered β-galactosidase genes. Arrows indicate Blue (β-galactosidase positive) cells.

5   **Figure 2:** Schematic representation of sequence alterations of the β-galactosidase gene produced by MMR defective host cells

Figure 3: Schematic representation of sec-hist secretion proteins for screening of structurally altered antigenic polypeptides and the sec-hist expression cassette (SEQ ID NO:17). Panel A: Schematic representation of sec-hist protein; Panel B: Sequence of sec-hist

10   expression cassette. In Panel B, the italic bold sequence represents a HindIII site for subcloning; the double underlined sequence on the 5' end represents leader sequence from the human IL-2; the underlined sequence on the 3' end represents the poly histidine sequence followed by 2 termination codons; sequence in capital letters represents sequence from the polylinker region of pUC18; the polylinker contains the following restriction enzymes for

15   cloning cDNAs: SacI-SacII-NotI-XbaI-SpeI-BamHI-SmaI-PstI-EcoRI.

Figure 4: Schematic diagram for high throughput screening of conditioned medium from TK clones for the identification of antigenic sec-hist polypeptides. Assays employ an *in vitro* antigenicity test using splenocytes from naïve mice (non-primed) and *antigen*-exposed (primed) mice. Clones exhibiting positive CM are then genetically analyzed to confirm

20   structural alterations within the sec-hist sequence, followed by protein purification and retesting of purified proteins. Purified proteins with the best stimulatory activity are then screened *in vivo* for immunogenicity. The screening assay can be repeated for several rounds to add additional alterations within the antigen (long arrow).

25   The inventors have discovered a method for developing hypermutable cells producing therapeutic antigens by taking advantage of the conserved mismatch repair (MMR) process of host cells. Dominant negative alleles of such genes, when introduced into cells or transgenic animals, increase the rate of spontaneous mutations by reducing the effectiveness of DNA repair and thereby render the cells or animals hypermutable.

Hypermutable cells or animals can then be utilized to develop new mutations in a gene or genes of interest. Blocking MMR in cells producing antigens (including, but not limited to, mammalian cells, plant cells, yeast cells, and prokaryotic cells) can enhance the rate of mutation within the gene encoding for the antigen that can be screened to identify clones

5　producing structurally altered polypeptides with enhanced antigenicitiy and immunogenicity.

In one aspect of the invention, the methods are useful for the production of antigens that have increased antigenicity and/or immunogenicity. Such antigens may be used as immunogens to elicit immune responses in animals against these antigens.

10　The antigens may be derived from, for example, pathogenic organisms or cancer cells such that an immune response is directed against the pathogenic organism or cancer cell and exerts an effect on the organism or cancer cell. The effect may be, for example, to prevent, inhibit or terminate the growth of the pathogenic organism or cancer cell when an immunogenic amount of the antigen is administered to an animal.

15　The pathogenic organisms from which antigens may be derived include bacteria, fungi, parasitic protozoa, helminths, and viruses. Non-limiting examples include species of the following genera: *Staphylococcus, Streptococcus, Bacillus, Bordetella, Clostridium, Escherichia, Haemophilus, Helicobacter, Klebsiella, Listeria, Salmonella, Vibrio, Yersinia, Neisseria, Treponema, Borrelia, Corynebacterium, Mycobacterium, Mycoplasma,*

20　*Chlamydia, Acremonium, Aspergillus, Blastomyces, Candida, Acanthamoeba, Ascaris, Babesia, Cryptosporidium, Echinococcus, Entamoeba, Giardia, Necator, Ancylostoma, Unicinaria, Leishmania, Onchocerca, Plasmodium, Schistosoma, Strongyloides, Taenia, Toxoplasma, Trichinella, Trichomonas, Trichuris, Trypanosoma, Dirofilaria, Brugia, Wuchereria,* and *Eimeria.* Non-limiting examples of viruses include adenovirus,

25　arborviruses, coronavirus, cytomegalovirus, enteroviruses, Epstein-Barr virus, hepatitis viruses, herpes viruses, immunodeficiency viruses (*e.g.*, HIV, FIV SIV), papilloma viruses, T-cell leukemia viruses, influenza viruses, mumps viruses, parainfluenzae viruses, parvoviruses, poxviruses, Rabies virus, respiratory syncytial virus, rhinoviruses, rotaviruses, Rubella viruses, and varicella-zoster viruses.

The antigens derived from the pathogenic organisms, for example, may be antigens known to elicit an immune response, for which an enhanced immune response is desired, or the antigen may be one that is known to generate a weak response for which an enhanced response is desired. It is also possible that some antigens that did not previously elicit an

5   immune response will become antigenic as a result of the methods of the invention and the phenomenon of hypermutability of cells which contain dominant negative alleles of mismatch repair genes.

The antigens produced by the method of the invention are novel immunogens that may be administered in an appropriate pharmaceutical carrier, such as an adjuvant, for

10   administration to animals as a vaccine. The antigens of the invention may be administered to animals in immunogenic amounts such that an antibody and/or a cell-mediated immune response is elicited. The administration of the antigens of the invention may be administered as a single dose, or, preferably as a plurality of doses to effect a boosted immune response. The route of administration may be any accepted route of immunization including, for

15   example, oral, intrmuscular, intrperitoneal, subcutaneous, intradermal, intranasal, or transdermal.

Doses for humans can readily be extrapolated from animal studies as taught by Katocs et al., Chapter 27 of REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Gennaro (Ed.) Mack Publishing Co., Easton, PA, 1990. Immunogenic dosages can be

20   adjusted by one skilled in the art, and may vary depending on several factors, including the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy, if required, and the nature and scope of the desired effect(s) (Nies et al., Chapter 3, GOODMAN & GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS, 9th Ed., Hardman et al., Eds.,

25   McGraw-Hill, New York, NY, 1996). Typically, an immunogenic amount of the antigens of the invention will be in the range of about 5 to about 100 μg.

The antigens of the present invention may be administered as single antigens or may be administered as combinations of antigens. As a non-limiting example, the antigen combinations may be antigens of the same pathogenic organism, or may be antigens of

different pathogenic organisms, such that immune responses are elicited to more than one pathogenic organism.

The antigens of the present invention are hypermutated by the methods of the invention which take advantage of the mismatch repair system. The process of MMR, also

5   called mismatch proofreading, is carried out by protein complexes in cells ranging from bacteria to mammalian cells. A MMR gene is a gene that encodes for one of the proteins of such a mismatch repair complex. Although not wanting to be bound by any particular theory of mechanism of action, a MMR complex is believed to detect distortions of the DNA helix resulting from non-complementary pairing of nucleotide bases. The non-complementary base

10   on the newer DNA strand is excised, and the excised base is replaced with the appropriate base, which is complementary to the older DNA strand. In this way, cells eliminate many mutations that occur as a result of mistakes in DNA replication.

Dominant negative alleies cause a MMR defective phenotype even in the presence of a wild-type allele in the same cell. An example of a dominant negative allele of a MMR gene

15   is the human gene *hPMS2-134*, which carries a truncating mutation at codon 134. The mutation causes the product of this gene to abnormally terminate at the position of the 134th amino acid, resulting in a shortened polypeptide containing the N-terminal 133 amino acids. Such a mutation causes an increase in the rate of mutations, which accumulate in cells after DNA replication. Expression of a dominant negative allele of a mismatch repair gene results

20   in impairment of mismatch repair activity, even in the presence of the wild-type allele. Any allele that produces such effect can be used in this invention.

Dominant negative alleles of a MMR gene can be obtained from the cells of humans, animals, yeast, bacteria, or other organisms (Prolla T.A. *et al.* (1994) *Science* 264:1091-1093; Strand M. *et al.* (1993) *Nature* 365:274-276; Su, S.S. *et al.* (1988) *J.*

25   *Biol. Chem.* 263:6829-6835). Such alleles can be identified by screening cells for defective MMR activity. Cells from animals or humans with cancer can be screened for defective mismatch repair. Cells from colon cancer patients may be particularly useful. Genomic DNA, cDNA, or mRNA from any cell encoding a MMR protein can be analyzed for variations from the wild type sequence. Dominant negative alleles of a MMR gene can also

be created artificially, for example, by producing variants of the *hPMS2-134* allele or other

MMR genes. Various techniques of site-directed mutagenesis can be used. The suitability of

such alleles, whether natural or artificial, for use in generating hypermutable cells or animals

can be evaluated by testing the mismatch repair activity caused by the allele in the presence

5   of one or more wild-type alleles, to determine if it is a dominant negative allele.

A cell or an animal into which a dominant negative allele of a mismatch repair gene

has been introduced will become hypermutable. This means that the spontaneous mutation

rate of such cells or animals is elevated compared to cells or animals without such alleles.

The degree of elevation of the spontaneous mutation rate can be at least 2-fold, 5-fold, 10-

10   fold, 20-fold, 50-fold, 100-fold, 200-fold, 500-fold, or 1000-fold that of the normal cell or

animal. The use of chemical mutagens such as but limited to methane sulfonate, dimethyl

sulfonate, O6-methyl benzadine, MNU, ENU, etc. can be used in MMR defective cells to

increase the rates an additional 10 to 100 fold that of the MMR deficiency itself.

According to one aspect of the invention, a polynucleotide encoding for a dominant

15   negative form of a MMR protein is introduced into a cell. The gene can be any dominant

negative allele encoding a protein, which is part of a MMR complex, for example, *PMS2,*

PMS1, MLH1, or *MSH2*. The dominant negative allele can be naturally occurring or made in

the laboratory. The polynucleotide can be in the form of genomic DNA, cDNA, RNA, or a

chemically synthesized polynucleotide.

20   The polynucleotide can be cloned into an expression vector containing a constitutively

active promoter segment [such as but not limited to CMV, SV40, Elongation Factor (EF) or

LTR sequences] or to inducible promoter sequences such as the steroid inducible pIND vector

(InVitrogen), tetracycline, or MMTV, where the expression of the dominant negative MMR

gene can be regulated. The polynucleotide can be introduced into the cell by transfection.

25   According to another aspect of the invention, a gene, a set of genes or a chimeric gene

encoding for whole or parts of a therapeutic antigen can be transfected into MMR deficient

cell hosts, the cell is grown and screened for clones containing genetically altered genes

encoding for antigens with new biochemical features including but not limited to increased

antigenicity. MMR defective cells may be of human, primates, mammals, rodent, plant, yeast or of the prokaryotic kingdom.

Transfection is any process whereby a polynucleotide is introduced into a cell. The process of transfection can be carried out in a living animal, *e.g.*, using a vector for gene

5 therapy, or it can be carried out *in vitro*, *e.g.*, using a suspension of one or more isolated cells in culture. The cell can be any type of eukaryotic cell, including, for example, cells isolated from humans or other primates, mammals or other vertebrates, invertebrates, and single celled organisms such as protozoa, yeast, or bacteria.

In general, transfection will be carried out using a suspension of cells, or a single cell,

10 but other methods can also be applied as long as a sufficient fraction of the treated cells or tissue incorporates the polynucleotide so as to allow transfected cells to be grown and utilized. The protein product of the polynucleotide may be transiently or stably expressed in the cell. Techniques for transfection are well known. Available techniques for introducing polynucleotides include but are not limited to electroporation, transduction, cell fusion, the

15 use of calcium chloride, and packaging of the polynucleotide together with lipid for fusion with the cells of interest. Once a cell has been transfected with the dominant negative MMR gene, the cell can be grown and reproduced in culture. If the transfection is stable, such that the gene is expressed at a consistent level for many cell generations, then a cell line results.

An isolated cell is a cell obtained from a tissue of humans or animals by mechanically

20 separating out individual cells and transferring them to a suitable cell culture medium, either with or without pretreatment of the tissue with enzymes, *e.g.*, collagenase or trypsin. Such isolated cells are typically cultured in the absence of other types of cells. Cells selected for the introduction of a dominant negative allele of a mismatch repair gene may be derived from a eukaryotic organism in the form of a primary cell culture or an immortalized cell line, or

25 may be derived from suspensions of single-celled organisms.

A polynucleotide encoding for a dominant negative form of a MMR protein can be introduced into the genome of an animal by producing a transgenic animal. The animal can be any species for which suitable techniques are available to produce transgenic animals. For example, transgenic animals can be prepared from domestic livestock, *e.g.*, bovine, swine,

sheep, goats, horses, etc.; from animals used for the production of recombinant proteins, *e.g.*, bovine, swine, or goats that express a recombinant polypeptide in their milk; or experimental animals for research or product testing, *e.g.*, mice, rats, guinea pigs, hamsters, rabbits, etc. Cell lines that are determined to be MMR defective can then be used as a source for

5   producing genetically altered genes encoding for therapeutic antigens *in vitro* by introducing whole, intact genes and/or chimeric genes encoding for a therapeutic antigen(s) into MMR defective cells from any tissue of the MMR defective animal.

Once a transfected cell line or a colony of transgenic animals has been produced, it can be used to generate new mutations in one or more gene(s) of interest. A gene of interest

10   can be any gene naturally possessed by the cell line or transgenic animal or introduced into the cell line or transgenic animal. An advantage of using such cells or animals to induce mutations is that the cell or animal need not be exposed to mutagenic chemicals or radiation, which may have secondary harmful effects, both on the object of the exposure and on the workers. However, chemical mutagens may be used in combination with MMR deficiency,

15   which renders such mutagens less toxic due to an undetermined mechanism. Hypermutable animals can then be bred and selected for those producing genetically variable cells that may be isolated and cloned to identify new cell lines that are useful for producing structurally altered polypeptides. Once an altered polypeptide is identified, the dominant negative MMR gene allele can be removed by directly knocking out the allele by technologies used by those

20   skilled in the art or by breeding to mates lacking the dominant negative allele to select for offspring with a desired trait and a stable genome. Another alternative is to use a CRE-LOX expression system, whereby the dominant negative allele is spliced from the animal genome once an animal containing a genetically diverse protein profile has been established. Yet another alternative is the use of inducible vectors such as the steroid induced pIND

25   (InVitrogen) or pMAM (Clonetech) vectors which express exogenous genes in the presence of corticosteroids.

Mutations can be detected by analyzing for alterations in the genotype of the cells or animals, for example by examining the sequence of genomic DNA, cDNA, messenger RNA, or amino acids associated with the gene of interest. Mutations can also be detected by

screening for the production of antigenicity. A mutant polypeptide can be detected by identifying alterations in electrophoretic mobility, spectroscopic properties, or other physical or structural characteristics of a protein encoded by a mutant gene. One can also screen for altered function of the protein *in situ*, in isolated form, or in model systems. One can screen

5   for alteration of any property of the cell or animal associated with the function of the gene of interest, such as but not limited to antigenicity.

According to another aspect of the invention, a high throughput mammalian cell-based assay is presented. A MMR defective cell line is transfected with a secretion cassette containing a leader sequence for secretion at the N-terminus fused to the target antigen. Cells

10   are grown and clones are plated by limiting dilution into microtitre plates and conditioned medium are screened for antigenic peptides. The advantage of such an approach is that the antigen is more similar to the natural polypeptide than it would be if produced by bacterial, yeast or baculovirus systems which tend to cause misfolding and/or distorted post-translational modifications.

15   Examples of mismatch repair proteins and nucleic acid sequences include the following:

PMS2 (mouse) (SEQ ID NO:5)

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       MEQTEGVSTE CAKAIKPIDG KSVHQICSGQ VILSLSTAVK ELIENSVDAG ATTIDLRLKD  60
20     YGVDLIEVSD NGCGVEEENF EGLALKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
       TISTCHGSAS VGTRLVFDHN GKITQKTPYP RPKGTTVSVQ HLFYTLPVRY KEFQRNIKKE 180
       YSKMVQVLQA YCIISAGVRV SCTNQLGQGK RHAVVCTSGT SGMKENIGSV FGQKQLQSLI 240
       PFVQLPPSDA VCEEYGLSTS GRHKTFSTFR ASFHSARTAP GGVQQTGSFS SSIRGPVTQQ 300
       RSLSLSMRFY HMYNRHQYPF VVLNVSVDSE CVDINVTPDK RQILLQEEKL LLAVLKTSLI 360
25     GMFDSDANKL NVNQQPLLDV EGNLVKLHTA ELEKPVPGKQ DNSPSLKSTA DEKRVASISR 420
       LREAFSLHPT KEIKSRGPET AELTRSFPSE KRGVLSSYPS DVISYRGLRG SQDKLVSPTD 480
       SPGDCMDREK IEKDSGLSST SAGSEEEFST PEVASSFSSD YNVSSLEDRP SQETINCGDL 540
       DCRPPGTGQS LKPEDHGYQC KALPLARLSP TNAKRFKTEE RPSNVNISQR LPGPQSTSAA 600
       EVDVAIKMNK RIVLLEFSLS SLAKRMKQLQ HLKAQNKHEL SYRKFRAKIC PGENQAAEDE 660
30     LRKEISKSMF AEMEILGQFN LGFIVTKLKE DLFLVDQHAA DEKYNFEMLQ QHTVLQAQRL 720
       ITPQTLNLTA VNEAVLIENL EIFRKNGFDF VIDEDAPVTE RAKLISLPTS KNWTFGPQDI 780
       DELIFMLSDS PGVMCRPSRV RQMFASRACR KSVMIGTALN ASEMKKLITH MGEMDHPWNC 840
       PHGRPTMRHV ANLDVISQN                                             859
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PMS2 (mouse cDNA) (SEQ ID NO:6)

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      gaattccggt gaaggtcctg aagaatttcc agattcctga gtatcattgg aggagacaga 60
      taacctgtcg tcaggtaacg atggtgtata tgcaacagaa atgggtgttc ctggagacgc 120
      gtcttttccc gagagcggca ccgcaactct cccgcggtga ctgtgactgg aggagtcctg 180
5     catccatgga gcaaaccgaa ggcgtgagta cagaatgtgc taaggccatc aagcctattg 240
      atgggaagtc agtccatcaa atttgttctg ggcaggtgat actcagttta agcaccgctg 300
      tgaaggagtt gatagaaaat agtgtagatg ctggtgctac tactattgat ctaaggctta 360
      aagactatgg ggtggacctc attgaagttt cagacaatgg atgtggggta gaagaagaaa 420
      actttgaagg tctagctctg aaacatcaca catctaagat tcaagagttt gccgacctca 480
10    cgcaggttga aactttcggc tttcgggggg aagctctgag ctctctgtgt gcactaagtg 540
      atgtcactat atctacctgc cacgggtctg caagcgttgg gactcgactg gtgtttgacc 600
      ataatgggaa aatcacccag aaaactccct accccgacc taaggaacc acagtcagtg 660
      tgcagcactt attttataca ctacccgtgc gttacaaaga gtttcagagg aacattaaaa 720
      aggagtattc caaaatggtg caggtcttac aggcgtactg tatcatctca gcaggcgtcc 780
15    gtgtaagctg cactaatcag ctcggacagg ggaagcggca cgctgtggtg tgcacaagcg 840
      gcacgtctgg catgaaggaa aatatcgggt ctgtgtttgg ccagaagcag ttgcaaagcc 900
      tcattccttt tgttcagctg cccctagtg acgctgtgtg tgaagagtac ggcctgagca 960
      cttcaggacg ccacaaaacc ttttctacgt ttcgggcttc atttcacagt gcacgcacgg 1020
      cgccgggagg agtgcaacag acaggcagtt tttcttcatc aatcagaggc cctgtgaccc 1080
20    agcaaaggtc tctaagcttg tcaatgaggt tttatcacat gtataaccgg catcagtacc 1140
      catttgtcgt ccttaacgtt tccgttgact cagaatgtgt ggatattaat gtaactccag 1200
      ataaaaggca aattctacta caagaagaga agctattgct ggccgtttta aagacctcct 1260
      tgataggaat gtttgacagt gatgcaaaca agcttaatgt caaccagcag ccactgctag 1320
      atgttgaagg taacttagta aagctgcata ctgcagaact agaaaagcct gtgccaggaa 1380
25    agcaagataa ctctccttca ctgaagagca cagcagacga gaaaagggta gcatccatct 1440
      ccaggctgag agaggccttt tctcttcatc ctactaaaga gatcaagtct aggggtccag 1500
      agactgctga actgacacgg agttttccaa gtgagaaaag gggcgtgtta tcctcttatc 1560
      cttcagacgt catctcttac agaggcctcc gtggctcgca ggacaaattg gtgagtccca 1620
      cggacagccc tggtgactgt atggacagag agaaaataga aaaagactca gggctcagca 1680
30    gcacctcagc tggctctgag gaagagttca gcaccccaga agtggccagt agctttagca 1740
      gtgactataa cgtgagctcc ctagaagaca gaccttctca ggaaaccata aactgtggtg 1800
      acctggactg ccgtcctcca ggtacaggac agtccttgaa gccagaagac catggatatc 1860
      aatgcaaagc tctacctcta gctcgtctgt cacccacaaa tgccaagcgc ttcaagacag 1920
      aggaaagacc ctcaaatgtc aacatttctc aaagattgcc tggtcctcag agcacctcag 1980
35    cagctgaggt cgatgtagcc ataaaaatga ataagagaat cgtgctcctc gagttctctc 2040
      tgagttctct agctaagcga atgaagcagt tacagcacct aaaggcgcag aacaaacatg 2100
      aactgagtta cagaaaattt agggccaaga tttgccctgg agaaaaccaa gcagcagaag 2160
      atgaactcag aaaagagatt agtaaatcga tgtttgcaga gatggagatc ttgggtcagt 2220
      ttaacctggg atttatagta accaaactga aagaggacct cttcctggtg gaccagcatg 2280
40    ctgcggatga gaagtacaac tttgagatgc tgcagcagca cacggtgctc caggcgcaga 2340
```

```
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     gagtcagaca gatgtttgct tccagagcct gtcggaagtc agtgatgatt ggaacggcgc 2640
     tcaatgcgag cgagatgaag aagctcatca cccacatggg tgagatggac cacccctgga 2700
     actgccccca cggcaggcca accatgaggc acgttgccaa tctggatgtc atctctcaga 2760
     actgacacac cccttgtagc atagagttta ttacagattg ttcggtttgc aaagagaagg 2820
     ttttaagtaa tctgattatc gttgtacaaa aattagcatg ctgctttaat gtactggatc 2880
     catttaaaag cagtgttaag gcaggcatga tggagtgttc ctctagctca gctacttggg 2940
     tgatccggtg ggagctcatg tgagcccagg actttgagac cactccgagc cacattcatg 3000
     agactcaatt caaggacaaa aaaaaaagaa tattttgaa gccttttaaa aaaaaa     3056
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PMS2 (human) (SEQ ID NO:7)

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     MERAESSSTE PAKAIKPIDR KSVHQICSGQ VVLSLSTAVK ELVENSLDAG ATNIDLKLKD 60
     YGVDLIEVSD NGCGVEEENF EGLTLKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
     TISTCHASAK VGTRLMFDHN GKIIQKTPYP RPRGTTVSVQ QLFSTLPVRH KEFQRNIKKE 180
     YAKMVQVLHA YCIISAGIRV SCTNQLGQGK RQPVVCTGGS PSIKENIGSV FGQKQLQSLI 240
     PFVQLPPSDS VCEEYGLSCS DALHNLFYIS GFISQCTHGV GRSSTDRQFF FINRRPCDPA 300
     KVCRLVNEVY HMYNRHQYPF VVLNISVDSE CVDINVTPDK RQILLQEEKL LLAVLKTSLI 360
     GMFDSDVNKL NVSQQPLLDV EGNLIKMHAA DLEKPMVEKQ DQSPSLRTGE EKKDVSISRL 420
     REAFSLRHTT ENKPHSPKTP EPRRSPLGQK RGMLSSSTSG AISDKGVLRP QKEAVSSSHG 480
     PSDPTDRAEV EKDSGHGSTS VDSEGFSIPD TGSHCSSEYA ASSPGDRGSQ EHVDSQEKAP 540
     ETDDSFSDVD CHSNQEDTGC KFRVLPQPTN LATPNTKRFK KEEILSSSDI CQKLVNTQDM 600
     SASQVDVAVK INKKVVPLDF SMSSLAKRIK QLHHEAQQSE GEQNYRKFRA KICPGENQAA 660
     EDELRKEISK TMFAEMEIIG QFNLGFIITK LNEDIFIVDQ HATDEKYNFE MLQQHTVLQG 720
     QRLIAPQTLN LTAVNEAVLI ENLEIFRKNG FDFVIDENAP VTERAKLISL PTSKNWTFGP 780
     QDVDELIFML SDSPGVMCRP SRVKQMFASR ACRKSVMIGT ALNTSEMKKL ITHMGEMDHP 840
     WNCPHGRPTM RHIANLGVIS QN                                        862
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PMS2 (human cDNA) (SEQ ID NO:8)

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     cgaggcggat cgggtgttgc atccatggag cgagctgaga gctcgagtac agaacctgct 60
     aaggccatca aacctattga tcggaagtca gtccatcaga tttgctctgg gcaggtggta 120
     ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180
     aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240
     tgtggggtag aagaagaaaa cttcgaaggc ttaactctga acatcacac atctaagatt 300
     caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360
     tcactttgtg cactgagcga tgtcaccatt tctacctgcc acgcatcggc gaaggttgga 420
     actcgactga tgtttgatca caatgggaaa attatccaga aacccccta ccccgcccc 480
     agagggacca cagtcagcgt gcagcagtta ttttccacac tacctgtgcg ccataaggaa 540
```

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      tttcaaagga atattaagaa ggagtatgcc aaaatggtcc aggtcttaca tgcatactgt 600
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      cctgtggtat gcacaggtgg aagccccagc ataaaggaaa atatcggctc tgtgtttggg 720
      cagaagcagt tgcaaagcct cattcctttt gttcagctgc ccctagtga ctccgtgtgt 780
   5  gaagagtacg gtttgagctg ttcggatgct ctgcataatc ttttttacat ctcaggtttc 840
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      gaaaagccca tggtagaaaa gcaggatcaa tccccttcat taaggactgg agaagaaaaa 1260
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      aagcctcaca gcccaaagac tccagaacca agaaggagcc ctctaggaca gaaaaggggt 1380
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      tttatcgcag attttttatgt tttgaaagac agagtcttca ctaacctttt ttgttttaaa 2700
      atgaaacctg ctacttaaaa aaaatacaca tcacacccat ttaaaagtga tcttgagaac 2760
      cttttcaaac c                                                     2771
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  40 PMS1 (human) (SEQ ID NO:9)

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YVLDGSGHIL SQKPSHLGQG TTVTALRLFK NLPVRKQFYS TAKKCKDEIK KIQDLLMSFG 180
ILKPDLRIVF VHNKAVIWQK SRVSDHKMAL MSVLGTAVMN NMESFQYHSE ESQIYLSGFL 240
PKCDADHSFT SLSTPERSFI FINSRPVHQK DILKLIRHHY NLKCLKESTR LYPVFFLKID 300
VPTADVDVNL TPDKSQVLLQ NKESVLIALE NLMTTCYGPL PSTNSYENNK TDVSAADIVL 360
SKTAETDVLF NKVESSGKNY SNVDTSVIPF QNDMHNDESG KNTDDCLNHQ ISIGDFGYGH 420
CSSEISNIDK NTKNAFQDIS MSNVSWENSQ TEYSKTCFIS SVKHTQSENG NKDHIDESGE 480
NEEEAGLENS SEISADEWSR GNILKNSVGE NIEPVKILVP EKSLPCKVSN NNYPIPEQMN 540
LNEDSCNKKS NVIDNKSGKV TAYDLLSNRV IKKPMSASAL FVQDHRPQFL IENPKTSLED 600
ATLQIEELWK TLSEEEKLKY EEKATKDLER YNSQMKRAIE QESQMSLKDG RKKIKPTSAW 660
NLAQKHKLKT SLSNQPKLDE LLQSQIEKRR SQNIKMVQIP FSMKNLKINF KKQNKVDLEE 720
KDEPCLIHNL RFPDAWLMTS KTEVMLLNPY RVEEALLFKR LLENHKLPAE PLEKPIMLTE 780
SLFNGSHYLD VLYKMTADDQ RYSGSTYLSD PRLTANGFKI KLIPGVSITE NYLEIEGMAN 840
CLPFYGVADL KEILNAILNR NAKEVYECRP RKVISYLEGE AVRLSRQLPM YLSKEDIQDI 900
IYRMKHQFGN EIKECVHGRP FFHHLTYLPE TT   932

PMS1 (human) (SEQ ID NO:10)

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gttctcagat catcacttcg gtggtcagtg ttgtaaaaga gcttattgaa aactccttgg 180
atgctggtgc cacaagcgta gatgttaaac tggagaacta tggatttgat aaaattgagg 240
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acacctcaaa aataaatagt catgaagatc ttgaaaattt gacaacttac ggttttcgtg 360
gagaagcctt ggggtcaatt tgttgtatag ctgaggtttt aattacaaca agaacggctg 420
ctgataattt tagcacccag tatgttttag atggcagtgg ccacatactt tctcagaaac 480
cttcacatct tggtcaaggt acaactgtaa ctgctttaag attatttaag aatctacctg 540
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atctcctcat gagctttggt atccttaaac ctgacttaag gattgtcttt gtacataaca 660
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MSH2 (human) (SEQ ID NO:11)

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QGVIKYMGPA GAKNLQSVVL SKMNFESFVK DLLLVRQYRV EVYKNRAGNK ASKENDWYLA 120
YKASPGNLSQ FEDILFGNND MSASIGVVGV KMSAVDGQRQ VGVGYVDSIQ RKLGLCEFPD 180
NDQFSNLEAL LIQIGPKECV LPGGETAGDM GKLRQIIQRG GILITERKKA DFSTKDIYQD 240
LNRLLKGKKG EQMNSAVLPE MENQVAVSSL SAVIKFLELL SDDSNFGQFE LTTFDFSQYM 300
KLDIAAVRAL NLFQGSVEDT TGSQSLAALL NKCKTPQGQR LVNQWIKQPL MDKNRIEERL 360
NLVEAFVEDA ELRQTLQEDL LRRFPDLNRL AKKFQRQAAN LQDCYRLYQG INQLPNVIQA 420
LEKHEGKHQK LLLAVFVTPL TDLRSDFSKF QEMIETTLDM DQVENHEFLV KPSFDPNLSE 480
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- 18 -

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LREIMNDLEK KMQSTLISAA RDLGLDPGKQ IKLDSSAQFG YYFRVTCKEE KVLRNNKNFS 540
TVDIQKNGVK FTNSKLTSLN EEYTKNKTEY EEAQDAIVKE IVNISSGYVE PMQTLNDVLA 600
QLDAVVSFAH VSNGAPVPYV RPAILEKGQG RIILKASRHA CVEVQDEIAF IPNDVYFEKD 660
KQMFHIITGP NMGGKSTYIR QTGVIVLMAQ IGCFVPCESA EVSIVDCILA RVGAGDSQLK 720
GVSTFMAEML ETASILRSAT KDSLIIIDEL GRGTSTYDGF GLAWAISEYI ATKIGAFCMF 780
ATHFHELTAL ANQIPTVNNL HVTALTTEET LTMLYQVKKG VCDQSFGIHV AELANFPKHV 840
IECAKQKALE LEEFQYIGES QGYDIMEPAA KKCYLEREQG EKIIQEFLSK VKQMPFTEMS 900
EENITIKLKQ LKAEVIAKNN SFVNEIISRI KVTT                             934
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MSH2 (human cDNA) (SEQ ID NO:12)

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```

MLH1 (human) (SEQ ID NO:13)

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MSFVAGVIRR LDETVVNRIA AGEVIQRPAN AIKEMIENCL DAKSTSIQVI VKEGGLKLIQ  60
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DGKCAYRASY SDGKLKAPPK PCAGNQGTQI TVEDLFYNIA TRRKALKNPS EEYGKILEVV 180
GRYSVHNAGI SFSVKKQGET VADVRTLPNA STVDNIRSIF GNAVSRELIE IGCEDKTLAF 240
KMNGYISNAN YSVKKCIFLL FINHRLVEST SLRKAIETVY AAYLPKNTHP FLYLSLEISP 300
QNVDVNVHPT KHEVHFLHEE SILERVQQHI ESKLLGSNSS RMYFTQTLLP GLAGPSGEMV 360
KSTTSLTSSS TSGSSDKVYA HQMVRTDSRE QKLDAFLQPL SKPLSSQPQA IVTEDKTDIS 420
SGRARQQDEE MLELPAPAEV AAKNQSLEGD TTKGTSEMSE KRGPTSSNPR KRHREDSDVE 480
MVEDDSRKEM TAACTPRRRI INLTSVLSLQ EEINEQGHEV LREMLHNHSF VGCVNPQWAL 540
AQHQTKLYLL NTTKLSEELF YQILIYDFAN FGVLRLSEPA PLFDLAMLAL DSPESGWTEE 600
DGPKEGLAEY IVEFLKKKAE MLADYFSLEI DEEGNLIGLP LLIDNYVPPL EGLPIFILRL 660
ATEVNWDEEK ECFESLSKEC AMFYSIRKQY ISEESTLSGQ QSEVPGSIPN SWKWTVEHIV 720
YKALRSHILP PKHFTEDGNI LQLANLPDLY KVFERC                          756
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MLH1 (human) (SEQ ID NO:14)

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        cttggctctt ctggcgccaa aatgtcgttc gtggcagggg ttattcggcg gctggacgag 60
        acagtggtga accgcatcgc ggcggggga gttatccagc ggccagctaa tgctatcaaa 120
        gagatgattg agaactgttt agatgcaaaa tccacaagta ttcaagtgat tgttaaagag 180
        ggaggcctga agttgattca gatccaagac aatggcaccg ggatcaggaa agaagatctg 240
5       gatattgtat gtgaaaggtt cactactagt aaactgcagt cctttgagga tttagccagt 300
        atttctacct atggctttcg aggtgaggct ttggccagca taagccatgt ggctcatgtt 360
        actattacaa cgaaaacagc tgatggaaag tgtgcataca gagcaagtta ctcagatgga 420
        aaactgaaag cccctcctaa accatgtgct ggcaatcaag gacccagat cacggtggag 480
        gacctttttt acaacatagc cacgaggaga aaagctttaa aaaatccaag tgaagaatat 540
10      gggaaaattt tggaagttgt tggcaggtat tcagtacaca atgcaggcat tagtttctca 600
        gttaaaaaac aaggagagac agtagctgat gttaggacac tacccaatgc ctcaaccgtg 660
        gacaatattc gctccatctt tggaaatgct gttagtcgag aactgataga aattggatgt 720
        gaggataaaa ccctagcctt caaaatgaat ggttacatat ccaatgcaaa ctactcagtg 780
        aagaagtgca tcttcttact cttcatcaac catcgtctgg tagaatcaac ttccttgaga 840
15      aaagccatag aaacagtgta tgcagcctat ttgcccaaaa acacacaccc attcctgtac 900
        ctcagtttag aaatcagtcc ccagaatgtg gatgttaatg tgcaccccac aaagcatgaa 960
        gttcacttcc tgcacgagga gagcatcctg gagcgggtgc agcagcacat cgagagcaag 1020
        ctcctgggct ccaattcctc caggatgtac ttcacccaga ctttgctacc aggacttgct 1080
        ggcccctctg gggagatggt taaatccaca acaagtctga cctcgtcttc tacttctgga 1140
20      agtagtgata aggtctatgc ccaccagatg gttcgtacag attcccggga acagaagctt 1200
        gatgcatttc tgcagcctct gagcaaaccc ctgtccagtc agccccaggc cattgtcaca 1260
        gaggataaga cagatatttc tagtggcagg gctaggcagc aagatgagga gatgcttgaa 1320
        ctcccagccc ctgctgaagt ggctgccaaa aatcagagct tggaggggga tacaacaaag 1380
        gggacttcag aaatgtcaga gaagagagga cctacttcca gcaaccccag aaagagacat 1440
25      cgggaagatt ctgatgtgga aatggtggaa gatgattccc gaaaggaaat gactgcagct 1500
        tgtacccccc ggagaaggat cattaacctc actagtgttt tgagtctcca ggaagaaatt 1560
        aatgagcagg gacatgaggt tctccgggag atgttgcata ccactccttt cgtgggctgt 1620
        gtgaatcctc agtgggcctt ggcacagcat caaaccaagt tataccttct caacaccacc 1680
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30      ctcaggttat cggagccagc accgctcttt gaccttgcca tgcttgcctt agatagtcca 1800
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        cctatcttca ttcttcgact agccactgag gtgaattggg acgaagaaaa ggaatgtttt 2040
35      gaaagcctca gtaaagaatg cgctatgttc tattccatcc ggaagcagta catatctgag 2100
        gagtcgaccc tctcaggcca gcagagtgaa gtgcctggct ccattccaaa ctcctggaag 2160
        tggactgtgg aacacattgt ctataaagcc ttgcgctcac acattctgcc tcctaaacat 2220
        ttcacagaag atggaaatat cctgcagctt gctaacctgc ctgatctata caaagtcttt 2280
        gagaggtgtt aaatatggtt atttatgcac tgtgggatgt gttcttcttt ctctgtattc 2340
40      cgatacaaag tgttgtatca aagtgtgata tacaaagtgt accaacataa gtgttggtag 2400
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- 21 -

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cacttaagac ttatacttgc cttctgatag tattccttta tacacagtgg attgattata 2460
aataaataga tgtgtcttaa cata                                          2484
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hPMS2-134 (human) (SEQ ID NO:15)

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MERAESSSTE PAKAIKPIDR KSVHQICSGQ VVLSLSTAVK ELVENSLDAG ATNIDLKLKD 60
YGVDLIEVSD NGCGVEEENF EGLTLKHHTS KIQEFADLTQ VETFGFRGEA LSSLCALSDV 120
TISTCHASAK VGT                                                     133
```

hPMS2-134 (human cDNA) (SEQ ID NO:16)

```
cgaggcggat cgggtgttgc atccatggag cgagctgaga gctcgagtac agaacctgct 60
aaggccatca aacctattga tcggaagtca gtccatcaga tttgctctgg gcaggtggta 120
ctgagtctaa gcactgcggt aaaggagtta gtagaaaaca gtctggatgc tggtgccact 180
aatattgatc taaagcttaa ggactatgga gtggatctta ttgaagtttc agacaatgga 240
tgtggggtag aagaagaaaa cttcgaaggc ttaactctga aacatcacac atctaagatt 300
caagagtttg ccgacctaac tcaggttgaa acttttggct ttcgggggga agctctgagc 360
tcactttgtg cactgagcga tgtcaccatt tctacctgcc acgcatcggc gaaggttgga 420
acttga                                                            426
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The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1: Stable expression of dominant negative MMR genes in cells results in widespread mutations of a reporter gene and its encoded polypeptide.

Expression of a dominant negative allele in an otherwise MMR proficient cell could render these host cells MMR deficient (Nicolaides, N.C. *et al.* (1998) *Mol.Cell. Biol.* 18:1635-1641). The creation of MMR deficient cells can lead to the generation of genetic alterations throughout the entire genome of a host organisms offspring, yielding a population

of genetically altered offspring or siblings that may produce biochemicals with altered

properties. This patent application teaches of the use of dominant negative MMR genes in

antigen-producing cells, including but not limited to rodent, human, primate, yeast, insect,

and prokaryotic cells producing proteins that may serve as therapeutic antigens for

5   vaccination. The cell expression systems described above that are used to produce antigens

are well known by those skilled in the art of vaccine therapeutics.

To demonstrate the ability to create MMR defective mammalian cells using dominant

negative alleles of MMR genes, we first transfected a MMR proficient rodent cell line with an

expression vector containing the human the previously published dominant negative PMS2

10   mutant referred herein as PMS134 (cell line referred to as TKPMS134), or with no insert (cell

line referred to as TKvec). A fragment containing the PMS134 cDNA was cloned into the

pEF expression vector which contains the constitutively active elongation factor promoter

along with the neomycin resistance gene as selectable marker. The results showed that the

PMS134 mutant could exert a robust dominant negative effect, resulting in biochemical and

15   genetic manifestations of MMR deficiency. A brief description of the methods are provided

below.

A hallmark of MMR deficiency is the generation of unstable microsatellite repeats in

the genome of host cells. This phenotype is referred to as microsatellite instability (MI). MI

consists of deletions and/or insertions within repetitive mono-, di- and/or tri nucleotide

20   repetitive sequences throughout the entire genome of a host cell. Extensive genetic analysis

eukaryotic cells have found that the only biochemical defect that is capable of producing MI

is defective MMR. In light of this unique feature that defective MMR has on promoting MI,

it is now used as a biochemical marker to survey for lack of MMR activity within host cells.

A method used to detect MMR deficiency in eukaryotic cells is to employ a reporter

25   gene that has a polynucleotide repeat inserted within the coding region that disrupts its

reading frame due to a frame shift. In the case where MMR is defective, the reporter gene

will acquire random mutations (i.e. insertions and/or deletions) within the polynucelotide

repeat yielding clones that contain a functional reporter gene. An example of the ability to

alter desired genes via defective MMR comes from experiments using Syrian Hamster

fibroblasts (TK) cells (described above), where a mammalian expression construct containing a defective β-galactosidase gene (referred to as pCAR-OF) was transfected into TKPMS134 or TKvect cells as described above. The pCAR-OF vector consists of a β-galactosidase gene containing a 29-basepair poly-CA tract inserted at the 5' end of its coding region, which

5   causes the wild-type reading frame to shift out-of-frame. This chimeric gene is cloned into the pCEP4, which contains the constitutively cytomegalovirus (CMV) promoter upstream of the cloning site and also contains the hygromycin-resistance (HYG) gene that allows for selection of cells containing this vector. The pCAR-OF reporter cannot generate β-galactosidase activity unless a frame-restoring mutation (*i.e.*, insertion or deletion) arises

10   following transfection into a host. Another reporter vector called pCAR-IF contains a β-galactosidase in which a 27-bp poly-CA repeat was cloned into the same site as the pCAR-OF gene, but it is biologically active because the removal of a single repeat restores the open reading frame and produces a functional chimeric β-galactosidase polypeptide (not shown). The pCAR vectors also contain the neomycin resistance gene as selectable marker. In these

15   proof-of-concept studies, TKPMS134 and TKvect cells were transfected with the pCAR-OF reporter vector and selected for 17 days in neomycin plus hygromycin selection medium. After the 17th day, resistant colonies were stained for β-galactosidase production to determine the number of clones containing a genetically altered β-galactosidase gene. All conditions produced a relatively equal number of neomycin/hygromycin resistant cells, however, only

20   the cells expressing the PMS134 dominant negative allele (TKPMS134) contained a subset of clones that were positive for β-galactosidase activity (Table 1). This result was also observed using a similar experimental strategy with a MMR proficient human cell line (data not shown). Table 1 shows the data from these experiments, where cell colonies were stained *in situ* for β-galactosidase activity and scored for activity. Cells were scored positive if the

25   colonies turned blue in the presence of X-gal substrate and scored negative if colonies remained white. Analysis of triplicate experiments showed a significant increase in the number of β-galactosidase positive cells in the TKPMS134 cultures, while no β-galactosidase cells were seen in the control TKvect cells.

Table 1. Number of TKPMS134 and TKvect cells containing functional β-galactosidase gene as a result of MMR deficiency.			
Cells	**White Colonies**	**Blue Colonies**	**% Clones with altered B-gal**
TKvect	65 +/- 9	0	0/65 = 0%
TKPMS134	40 +/- 12	28 +/- 4	28/68 = 41%

Table 1. β-galactosidase expression of HBvec, HBPMS2 and HB134 cells transfected with pCAR-OF reporter vectors. Cells were transfected with the pCAR-OF β-galactosidase reporter plasmid. Transfected cells were selected in hygromycin and G418, expanded and stained with X-gal solution to measure for β-galactosidase activity (blue colored cells). 3 plates each were analyzed by microscopy. The results below represent the mean +/- standard deviation of these experiments.

TKPMS134/pCAR-OF clones that were pooled and expanded also showed a number of cells that contained a functional β-galactosidase gene. No β-galactosidase positive cells were observed in TKvect cells transfected with the pCAR-OF vector. These data are shown in Figure 1 where the dark staining in panel B represent β-galactosidase positive cells present in the TKPMS134/pCAR-OF cultures while none are found in the TKvect cells grown under similar conditions (panel A). These data demonstrate the ability of dominant negative alleles of MMR genes to generate *in vivo* gene alterations, which allows for the rapid screening of clones with altered polypeptides exhibiting new biochemical features.

To confirm that alterations within the nucleotide sequences of the β-galactosidase gene was indeed responsible for the *in vivo* β-galactosidase activity present in TKPMS134 clones, RNA was isolated from TKPMS134/pCAR-OF and TKvect/pCAR-OF and the β-galactosidase mRNA primary structure was examined by reverse transcriptase polymerase chain reaction (RT-PCR) amplification and sequencing. Sequence analysis of β-galactosidase message from TKvect cells found no structural alterations in the input gene sequence. Analysis of the β-galactosidase message from TKPMS134 cells found several changes within the coding sequences of the gene. These sequence alterations included insertion and deletions of the poly-CA tract in the amino terminus as expected. Other

alterations included insertions of sequences outside of the poly-CA repeat as well as a series of single base alterations (transversions and transitions) contained throughout the length of the gene.

5   ***In situ* X-gal staining**

For *in situ* analysis, 100,000 cells are harvested and fixed in 1% gluteraldehyde, washed in phosphate buffered saline solution and incubated in 1 ml of X-gal substrate solution [0.15 M NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_6$, 3.3 mM $K_3Fe(CN)_6$, 0.2% X-Gal] in 24 well plates for 2 hours at 37°C. Reactions are stopped in 500 mM sodium bicarbonate

10   solution and transferred to microscope slides for analysis. Three plates each are counted for blue (β-galactosidase positive cells) or white (β-galactosidase negative cells) to assess for MMR inactivation. Table 1 shows the results from these studies.

EXAMPLE 2: Generation of an expression cassette for screening of structurally altered polypeptides in MMR defective cells.

15   In order to produce recombinant proteins for screening of highly antigenic polypeptides, a fusion gene cassette was engineered that encodes for a secreted polypeptide containing a six polyhistidine domain at the C-terminus, which is useful for purification. This gene cassette is referred to as sec-hist. This gene was constructed by PCR using DNA from the pUC18 plasmid as template. The sense primers contained nucleotide sequences

20   corresponding to the leader sequence of human interleukin-2 (ref 32), which has been found to produce robust amounts of secreted polypeptides from TK cells (personal observation). This domain was introduced at the 5' end of the pUC18 polylinker. Antisense primers containing nucleotide sequences encoding for 6 histidines were used to position these residues at the 3' end of the pUC18 polylinker. The nucleotide sequence of these primers are

25   listed below.

SENSE Primer:

5'*aagctt*ccatgtacaggatgcaactcctgtcttgcattgcactaagtcttgcacttgtcacaaacagtgcaCAAAAGCTGGA GCTC-3' (SEQ ID NO:1)

The italic sequence represents a HindIII site for subcloning. The underlined sequence represents leader sequence from the human IL-2. Sequence in capital letters represents sequence from the start of the polylinker region of pUC18.

5   **ANTISENSE Primer:**

5'*ccggatcc*ctactagtggtgatggtgatggtgGCTTGATATCGAATTCCTG-3' (SEQ ID NO:2)

The italic sequence represents a HindIII site for subcloning. The underlined sequence represents 6 codons encoding for histidine residues followed by 2 termination codons. Sequence in capital letters represents sequence to the 3' end of the pUC18 polylinker.

10   Amplified products were obtained using buffer conditions as previously described . Amplification reactions were carried out at 94°C for 30 sec, 52°C for 2 min, and 72°C for 2 min for 25 cycles. Products were run on 1% agarose gels containing ethidium bromide, and products of the expected molecular weight were excised and purified by Gene Clean (Bio101). Products were then cloned into T-tailed vectors (InVitrogen) as suggested by the

15   manufacturer. Recombinant clones were analyzed by restriction analysis and by DNA sequencing. Several clones contained fragments with the expected genomic sequence. The parental clone is referred to as TAsec-hist.

A schematic diagram of the sec-hist fusion protein is shown in Figure 3A

In order to generate TK cells that secrete the sec-hist polypeptide, the TAsec-hist plasmid is

20   digested with HindIII to release the sec-hist insert. The insert was cloned into the unique HindIII site of the pCEP4 mammalian expression vector, which also contains the Hygromycin resistance gene as selectable marker. Recombinant clones were analyzed by restriction digest and sequencing to assure the authenticity of the construct.

Inserts can now be designed via PCR or direct cloning using the restricition sites

25   contained within the polylinker (see Figure 3B).

Recombinant pCEPsec-hist plasmid will then be transfected into TK cells as previously described using cationic lipids. Cells will be cotransfected along with the pEFPMS134, which is a mammalian expression vector containing the PMS134 dominant negative MMR gene allele under control of the constitutive elongation factor (EF) promoter.

This vector contains the neomycin resistance gene and allows for double selection of TK cells for both the sec-hist and pEFPMS134 vectors. TK cells will also be cotransfected with the sec-hist and pEF empty vector as a control.

Cells are co-selected for 14 days in 0.6 mg/ml G418 and 0.8mg/ml hygromycin B
5   (these concentrations have been previously determined for double transfection of TK cells). After 14 days, macroscopic colonies will be isolated and subcloned into 24 well dishes (Nunc) as 1 ml cultures. Clones will then be analyzed for secreted sec-hist protein using both ELISA and western blot analysis of conditioned supernatants from sec-hist/pEFPMS134, sec-hist/pEFempty vector, and parental TK cells. A monoclonal anti-HIS antibody (Santa Cruz),
10 which has been successfully used for other western and ELISA studies, will be used for both assays. Analysis of PMS134 expression will be determined by western blot using a PMS2-specific polyclonal antibody (Morphotek, personal communication).

ELISA will be performed on conditioned medium (CM) from TK cells transfected with pCEP4sec-hist to screen for high producers of the sec-hist polypeptide. ELISAs are
15 carried out as follows. Twohundred microliter aliquots of conditioned medium are taken from pCEP4sec-hist transfected and control cells. Aliquots are placed into 1.5ml eppendorf tubes and centrifuged at 14,000Xg for 3 minutes to pellet cell debris. Supernates are then collected and 50 µls are placed into triplicate wells of a 96-well polystyrene microtiter plate (Nunc). Plates are incubated at room temperature for 4 hours, washed twice with 200 µls of
20 1X Phosphate Buffered Saline (PBS) solution, pH 7.0 (Life Technologies), and blocked with 100 µls of 5%milk in 1X PBS for 1 hour. Plates are then incubated with a monoclonal anti-HIS antibody (diluted 1:1000 in 1X PBS) (Santa Cruz) for 2 hours at room temperature and then washed twice with 200 µls of 1X PBS, and probed with an anti-mouse-horse radish peroxidase (HRP) conjugated secondary antibody diluted 1:3000 in PBS. Plates are then
25 incubated at room temperature for 1 hour, washed three times with 200 µls of 1X PBS and incubated with TMB substrate (BioRad) for 15-30 minutes. After incubation is completed, reactions are stopped using 0.1N H_2SO_4 and plates are read using a BioRad microplate reader at 415nm. Clones are determined to be positive for secreted sec-hist if expected cells are found to produce a significant signal over control cells. Conditioned medium from positive

cultures will then be analyzed by western blot using the anti-HIS antibody as probe to confirm ELISA data.

Western blot analysis will be carried out as follows. Briefly, 50μls of CM or 50,000 cells from each culture is directly lysed in 2X lysis buffer (60 mM Tris, pH 6.8, 2% SDS,

5   10% glycerol, 0.1 M 2-mercaptoethanol, 0.001% bromophenol blue) and samples are boiled for 5 minutes. Lysate proteins are separated by electrophoresis on 4-20% Tris glycine gels (Novex) and then electroblotted onto Immobilon-P (Millipore) in 48 mM Tris base, 40 mM glycine, 0.0375% SDS, 20% methanol and blocked overnight at 4°C in Tris-buffered saline plus 0.05% Tween-20 and 5% condensed milk. Filters are then probed with an antibody

10   generated against the PMS134 or the polyHIS tag (Santa Cruz), followed by a secondary HRP-conjugated antibody. After incubation with the secondary antibody, blots are developed using chemiluminescence (Pierce) and exposed to film to determine PMS134 and sec-hist expression. Clones exhibiting expression of both genes will then be used in experiments described above.

15   A potential technical problem may exist in expressing the sec-hist protein due to toxicity that it may have on the growth of TK cells. If the production of no or low amounts of sec-hist polypeptide is found to occur in the above analysis, a HindIII sec-hist fragment from the TAsec-hist plasmid will be subcloned into the unique HindIII site of the pIND/V5 steroid inducible vector (Invitrogen). This vector has been found to produce robust protein

20   expression in TK cells upon in steroid induction. This application teaches the use of employing an inducible vector containing the sec-hist expression cassette to express polypeptides in TK cells that may be toxic under constitutively expressed conditions. Cells that are found to co-express the PMS134 and the sec-hist genes or the control cell expressing the pEF empty vector and the sec-hist gene are cultured under high growth

25   conditions in media containing neomycin, hygromycin and vitamins, which has been shown to increase the doubling time of TK cells and enhance the genetic alteration of β-galactosidase reporter plasmids *in vivo* (data not shown). Briefly, cells are grown in vitamin enriched medium for 20 doublings (~17 days), a time at which it has been found that 20-40% of clones contain sequence alterations within a particular genetic locus. After selection, cells

will be subcloned in 96-well microtiter plates by limiting dilutions. Clones will be grown for 5 days in the presence of neomycin/hygromycin-free medium containing heat inactivated serum to remove complement for *in vitro* antigenic assays that will be performed using murine lymphocytes as described in section below.

5   The sec-hist expression vector cassette can also be transfected into cell lines that are "naturally" defective for MMR such as the human cell lines derived from colon cancer tumors such as but not limited to HCT116 and DLD-1. The vector can be in the constitutive backbone pCEP4 or under control of the steroid-inducible vectors pIND or pMAM.

The techniques described above teach us the use of producing structurally altered

10   antigens from mammalian cells to ensure proper folding or post-translation modifications of the polypeptide. This approach gives an advantage over others that employ the use of prokaryotic, yeast or baculovirus produced antigens that have been found to produce weak antigenic responses due to misfolding or improper post-translational modifications.

15   **EXAMPLE 3: Screening strategy to identify cell clones producing highly antigenic polypeptides.**

In order to identify antigenic polypeptides produced by TKPMS134 cell clones, the following *in vitro* assays will be performed.

First, the lymphocyte stimulatory activity of sec-hist polypeptides will be measured

20   by adding CM of TKsec-hist cells with or without the PMS134 to lymphocytes derived from naïve or whole antigen exposed Balb/C mice. Briefly, 2 mice will be infected with whole antigen in the presence of Freund's Complete Adjuvant by subcutaneous injection in the tail with a 100-µl 1/1 mixture of complete Freund's adjuvant (CFA) (Difco). Two subcutaneous boosts will be performed with the same quantity of antigen, mixed 1/1 with incomplete

25   Freund's adjuvant (Difco), after 2 and 4 weeks. Two control mice will receive adjuvant alone. Mice are sacrificed 5 days after the second boost (at day 33). Peripheral blood mononuclear cells (PBMCs) from whole blood and splenocytes from spleens of will be harvested following the previously described procedures (Nicolaides, N.C. *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:13175-13180). For splenocyte assays, whole spleens are pressed through

sterile wire mesh into RPMI medium (Life Technology). Next, cells are washed twice in RPMI and incubated for 10 minutes in RBC lysis buffer. Cells are then washed again and resuspended at 1 X 10^5 cells/ml in RPMI-1640 medium plus 10% heat inactivated fetal bovine serum. One hundred microliters of cells are aliquoted into twenty 96-well titer flat

5   bottom plates.

For PBMC isolation, whole blood is isolated by eye puncture and collected into vacutainer tubes containing EDTA. An equal volume of PBS (Mg^{2+}/Ca^{2+}-free) is added to whole blood. PBMCs are isolated by centrifugation over Ficoll-Paque gradients (Pharmacia Biotech 17-1440-02). Purified cells are seeded at 1 X 10^5 cells/ml in RPMI 1640 containing

10   10% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and 100μls are plated in 96 well flat bottom microtiter plates and incubated at 37 °C in 5% CO_2.

To measure for T-cell activation, PBMCs and splenocytes from primed and non-primed mice are incubated with 10% conditioned medium (CM) from TKsec-hist cells with or without the PMS134 gene. 5 μg/ml of concavalinA (ConA) is used as a positive control

15   for splenocyte culture assays, while 5 μg/ml phorbol 12-myristate 13-acetate and 1 μg/ml phytohemagglutinin (Sigma) are used as a positive control for PBMC cultures. CM from parental TK cells grown in the presence of RPMI with 10% heat inactivated medium will be used as negative control. Previous studies using CM from TK cells have found no stimulatory activity to be produced on PBMCs or splenocytes (N. Nicolaides, personal

20   observation). Cultures are incubated at 37 °C in 5% CO_2 for 6 days and scored for antigenic activity as determined by proliferation assay. Proliferation is assayed using a modified protocol of the acid phosphatase assay as described (Grasso, L. *et al.* (1998) *J. Biol. Chem.* 273:24016-24024). Briefly, 50 μls of a buffer containing 0.1 M sodium acetate (pH 5.5), 0.1% Triton X-100, and 10 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate)

25   is added directly to each well containing 0.2 ml of growth medium and incubated for 1.5 h at room temperature. Reactions are terminated by the addition of 0.05 N sodium hydroxide and quantified by absorbance at 410 nm using a BioRad plate reader. Data is represented as a stimulation index (SI), which is the proliferation of experimental data points divided by the

mean of 10 aliquots of CM from TK parental cells. All experiments will be performed in at
least triplicate.

It is expected that several TKsec-hist clones co-expressing the PMS134 protein will
be found to have an enhanced antigenicity on PBMCs and/or splenocytes due to

5   conformational changes that will occur within the coding region of the target antigen. These
changes may form secondary domains that serve as T and/or B cell epitopes and in turn are
responsible for stimulating their respective activation. Clones that reproducibly produce
enhanced antigenic activation will be sequenced to confirm and identify that a structural
alteration(s) has indeed occurred within the coding region of the gene. Sequence data may

10   also shed additional light into the importance of critical domains within this candidate
vaccine polypeptide for additional rounds of alteration that may lead to the creation of a
super-antigen that may serve as a potent vaccine.

Sequence analysis of clones will be performed as follows. First positive clones will
be expanded from 96-well plates to 24-well plates. Confluent wells will be expanded and

15   cells will be harvested for RNA extraction. RNA extraction and reverse transcription will be
carried out using the Trizol method as previously described (Nicolaides, N.C. *et al.* (1997)
Proc. Natl. Acad. Sci. USA 94:13175-13180; Grasso, L. *et al.* (1998) *J. Biol. Chem.*
273:24016-24024). Reverse transcription will be carried out using Superscript II (Life
Technology) as previously described (Nicolaides, N.C. *et al.* (1998) *Mol. Cell. Biol.* 18:1635-

20   1641). cDNAs will be amplified to isolate the sec-hist transcript using the sense primer: 5'-
catgtacaggatgcaactcctg-3' (SEQ ID NO:3), which is located at the IL-2 leader sequence site
(see Figure 3), and the antisense primer: 5'-tactagtggtgatggtgatggtg-3' (SEQ ID NO:4),
which is located at the C-terminal polyhis site. Amplification is carried out at 94°C for 30
sec, 52°C for 2 min, 72°C for 2 min for 30 cycles. Reactions are analyzed on agarose gels. If

25   products of the expected molecular weight are generated then samples will be cleaned using
the QIAquick PCR template kit (Qiagen) to remove PCR amplimers and sequenced using the
following primers that cover the entire coding region of the sec-hist gene. Clones are then
sequenced using primers specific to the gene encoding the antigen.

Clones producing genetically altered sec-hist polypeptides will then be expanded into T-75 flasks to a density that will enable for the sufficient production of secreted sec-hist polypeptide in the CM. Conditioned medium containing the sec-hist polypeptide is then collected, and centrifuged at 3,500Xg for 10 minutes to remove cellular debris. CM is then

5   loaded onto a 10ml- HiTrap Nickel column following the manufacturer's protocol (Pharmacia). After absorption and washing, the column is treated with 200 mM imidazole to elute the fusion protein. Recovered polypeptides are then analyzed by SDS-PAGE using 4-12% NuPAGE gels (Novex) and silver stained following the manufacturer's protocol (Novex). Due to the random nature of defective MMR, the possibility exists that sec-hist

10 alleles may be generated by clones producing enhanced antigenic polypeptides, which contain a nonsense or frame-shift mutation, therefore forming polypeptides lacking a polyHIS C-terminus. If a nonsense or frameshift mutation does occur in clones producing polypeptides with increased antigenicity, then the new allele is reengineered via PCR to contain a polyHIS tag at the C-terminus, and this new fusion protein will be rescreened as

15 above.

Purified polypeptides will be rescreened at a final concentration of 10 µg/ml in the *in vitro* assays described above to confirm that the antigenic activity is indeed coming from the sec-hist protein. TK cells producing altered antigens with enhanced activity on both PBMCs and splenocytes will then be tested along with the non-altered sec-hist protein *in vivo* in

20 Balb/C mice for the ability to illicit an immune response in the absence of adjuvant. A schematic diagram outlining the screening procedure is given in Figure 4.

EXAMPLE 4: Screening strategy to identify cell clones producing highly immunogenic antigens.

25 To test the immunogenic potential that the altered polypeptides identified from EXAMPLE 3 have *in vivo*, Balb/C mice will be injected with the 6 most antigenic polypeptides and the wild type sec-hist polypeptide produced from TKEFempty/sec-hist cells in the absence of adjuvant. Briefly, mice will be immunized with 30 µgs of purified sec-hist protein in sterile phosphate buffered saline (PBS) without adjuvant by subcutaneous injection

in the tail. One group of mice will receive a 100μls of a mixture of polypeptides diluted 1/1

in complete Freund's adjuvant (CFA) (Difco) as a positive control. Two subcutaneous boosts

will be performed with the same quantity of antigen, and the mouse receiving the polypeptide

mixture with adjuvant will be boosted with a 1/1 mixture with incomplete Freund's adjuvant

5   (Difco), at 2 and 4 weeks after the initial injection. Mice will be bled 5 and 15 days after the

second boost (at day 33) to measure for antigen titers. Control mice will receive PBS alone.

Before the start of immunization, a prebleed will be obtained from each mouse.

Immune responses will be measured by screening for whole Ig and sec-hist specific

antibody titers by ELISA following the protocol described above. For antigen specific

10   antisera titers, 96-well plates will be coated with a 50uls of a solution containing 5μg/ml each

antigen used for vaccination in PBS. Plates will then be probed with serial dilutions of

prebleeds, 5 day and 15 day bleeds. Detection of antibodies will be done using a sheep anti-

mouse-HRP conjugated secondary antibody followed by incubation with TMB substrate as

described above.

15   Success of the strategy proposed in this program will be demonstrated with the

generation of antisera from mice immunized with altered sec-hist protein that is able to cross

react with the native sec-hist protein. If no titers are found in the samples without adjuvant,

mice will be administrated antigen in complete Freund's Adjuvant and titers analyzed 14

days later, followed by immunization in Incomplete Freund's at day 17 if no titers are found

20   and analyzed at day 31.

A potential problem that may occur with the outlined strategy is that antibody titers

may be generated against the sec-polyhistidine fusion domain. To determine if antisera is

able to identify authentic sec-his polypeptides, the antigen lacking the polyhistidine domain

will be generated by *in vitro* transcription-translation (TNT) in the presence of radiolabelled

25   methionine. A template containing the sec-hist polypeptide will also be made and the

encoded protein used as a control. Templates for the TNT reactions will be generated by PCR

as described (Nicolaides, N.C. *et al.* (1998) *Mol. Cell. Biol.*18:1635-1641). Briefly, the

TAsec-hist plasmid will be used as template to amplify gene segments that encode for the

untqagged antigen or the sec-hist tagged protein.

Translated polypeptides are first analyzed by SDS-PAGE gel electrophoresis and autoradiography to ensure that the polypeptide with the expected molecular weight are synthesized. Proteins are then immunoprecipitated using antiserum from vaccinated mice to determine if these antibodies recognize authentic antigen sequences. Briefly,

5   immunoprecipitations are performed on *in vitro*-translated proteins by mixing the translation reaction mixtures with 100μls of mouse antiserum or 1 μg of a HIS-specific monoclonal antibody (MAB) (Santa Cruz) in 400 μls of EBC buffer (50 mM Tris [pH 7.5], 0.1 M NaCl, 0.5% Nonidet P-40). After incubation for 1 h at 4°C, protein A-Sepharose (Sigma) is added to a final concentration of 10% and the reaction mixtures are incubated at 4°C for 1 h.

10   Proteins bound to protein A are washed five times in EBC and separated by electrophoresis on 4-20% Tris-glycine gradient gels, which are then dried and autoradiographed.

If antisera are able to react with authentic sequences lacking HIS residues, the data from these studies will be continued in further preclinical animal studies.

15   **Discussion**

The initial steps of MMR are dependent on two protein complexes, called MutSα and MutLα. The use of dominant negative MMR alleles are able to perturb the formation of these complexes with downstream biochemicals involved in the excision and polymerization of nucleotides comprising the "corrected" nucleotides. Examples from this application show

20   the ability of a truncated MMR allele (PMS134 is capable of blocking MMR resulting in a hypermutable cell line that gains genetic alterations throughout its entire genome per cell division. Once a cell line is produced that contains genetic alterations within genes encoding for an antigen, it is desirable to restore the genomic integrity of the cell host. This can be achieved by the use of inducible vectors whereby dominant negative MMR genes are cloned

25   into such vectors, introduced into antigen-producing cells and the cells are cultured in the presence of inducer molecules and/or conditions. Inducible vectors include but are not limited to chemical regulated promoters such as the steroid inducible MMTV, tetracycline regulated promoters, temperature sensitive MMR gene alleles, and temperature sensitive promoters.

The results described above lead to several conclusions. First, expression of PMS134 results in an increase in microsattelite instability in TK cells. That this elevated microsattelite instability is due to MMR deficiency was proven by evaluation of extracts from stably transduced cells and stability of a tract contained within the pCAR-OF vector. The

5   expression of PMS134 results in a polar defect in MMR, which was only observed using heteroduplexes designed to test repair from the 5' direction (no significant defect in repair from the 3' direction was observed in the same extracts). Interestingly, cells deficient in hMLH1 also have a polar defect in MMR, but in this case preferentially affecting repair from the 3' direction. It is known from previous studies in both prokaryotes and eukaryotes that the

10   separate enzymatic components mediate repair from the two different directions. These results strongly suggest a model in which 5' repair is primarily dependent on hPMS2 while 3' repair is primarily dependent on hMLH1. It is easy to envision how the dimeric complex between PMS2 and MLH1 might set up this directionality. The combined results also demonstrate that a defect in directional MMR is sufficient to produce a MMR defective

15   phenotype and suggests that any MMR gene allele is useful to produce genetically altered TK cells, or a cell line that is producing antigenic gene products. Moreover, the use of such MMR alleles will be useful for generating genetically altered polypeptides with altered structures as effective vaccine agents.

This application also teaches us that ANY method used to block MMR can be

20   performed to generate hypermutablility in an antigen-producing cell that can lead to genetically altered proteins with enhanced biochemical features such as but not limited to increased antigenicity, increased immunogenicity, and enhanced pharmacokinetic profiles.

The blockade of MMR in such cells can be through the use of dominant negative MMR gene alleles from any species including bacteria, yeast, protozoa, insects, rodents,

25   primates, mammalian cells, and man. Blockade of MMR can also be generated through the use of antisense RNA or deoxynucleotides directed to any of the genes involved in the MMR biochemical pathway. Blockade of MMR can be through the use of polypeptides that interfere with subunits of the MMR complex including but not limited to antibodies. Finally, the blockade of MMR may be through the use chemicals such as but not limited to

nonhydrolyzable ATP analogs, which have been shown to block MMR (Galio, L. *et al.* (1999) *Nucl. Acids Res.* 27:2325-2331; Spampinato, C. and P. Modrich (2000) *J. Biol. Chem.* 275:9863-9869).